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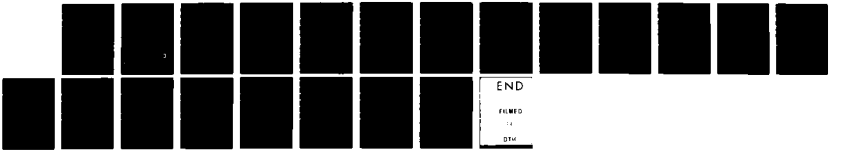

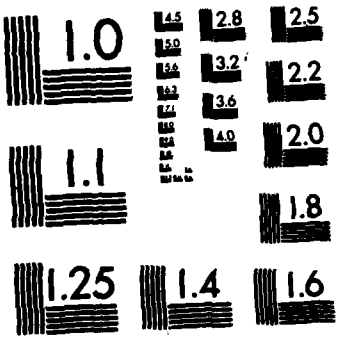

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*INSTITUTE REPORT NO. 135*

# EFFECT OF TOPICALLY APPLIED DIISOPROPYLFLUOROPHOSPHATE ON GLUCOSE METABOLISM IN THE RAT

*GEORGE J. KLAIN, PhD*
*and*
*JAMES J. SCHNEIDER, BS*

**DIVISION OF CUTANEOUS HAZARDS**
**LETTERMAN ARMY INSTITUTE OF RESEARCH**
**and**
**DEPARTMENT OF BIOLOGY**
**UNIVERSITY OF SAN FRANCISCO**





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**LETTERMAN ARMY INSTITUTE OF RESEARCH**
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Effect of Topically Applied Diisopropyfluorophosphate on Glucose Metabolism in the Rat--Klain and Schneider



In conducting the research described in this report, the investigation adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council.

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

A solution of DFP in peanut oil was applied topically on the clipped mid-lumbar region of rats and two hours later alterations in glucose utilization by skin, liver and adipose tissue preparations were determined. DFP had no demonstratable effect on glucose oxidation. In contrast, DFP enhanced fatty acid synthesis by 70% over the control values in the skin and by 56 and 92% in the liver and adipose tissue, respectively. DFP stimulated synthesis of cholesterol and sterols by 69% in the liver and doubled and tripled the

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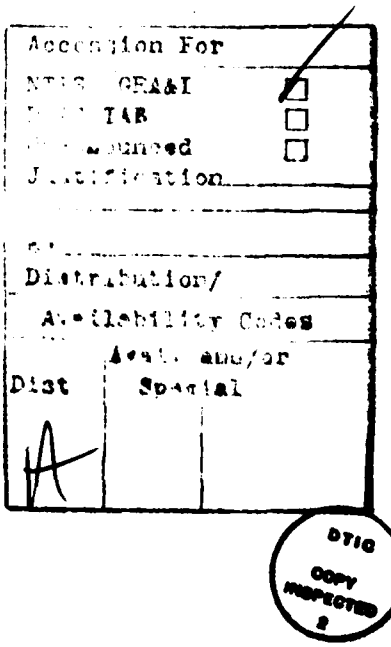

## ABSTRACT

A solution of diisopropylfluorophosphate in peanut oil was applied topically on the clipped mid-lumbar region of rats and two hours later alterations in glucose utilization by skin, liver, and adipose tissue preparations were determined. DFP had no demonstratable effect on glucose oxidation. In contrast, DFP enhanced fatty acid synthesis by 70% over the control values in the skin and by 56 and 92% in the liver and adipose tissue, respectively. DFP stimulated synthesis of cholesterol and sterols by 69% in the liver and doubled and tripled the synthesis, respectively, in the skin and adipose tissue. DFP effects on glycogen synthesis appear to be the result of a decreased activity of the key enzymes in the synthetic pathway. Enhanced synthesis of various lipid components appears to be substrate-induced.

## PREFACE

An affiliation program between Letterman Army Institute of Research (LAIR) and the University of San Francisco (USF) was established in 1980. Under this program qualified students from USF actively participate in research conducted at LAIR and their efforts are credited toward their course work at the University. Some of the data presented in this report were used by James J. Schneider in partial fulfillment of the requirement for graduate research in Biology 220 at USF.

TABLE OF CONTENTS

Effect of Topically Applied Diisopropylfluorophosphate on Glucose Metabolism in the Rat--Klain and Schneider

Organophosphate compounds (OPC) are known to disrupt nervous system function in animals and humans. A biocidal activity, first recognized in 1937, led to their development in Germany during World War II as potential warfare agents, e.g., the nerve gases soman, sarin and tabun (1). More recently OPC less toxic to humans have been developed and used widely as pest control agents, e.g., malathion and parathion. Their toxic properties appear related to an ability to inhibit serine proteases and cholinesterase throughout the central and peripheral nervous systems (2,3). All OPC share similar chemical structures and pharmacological actions. They differ mainly in regard to potency and use (4).

It is generally accepted that OPC are readily absorbed through the skin following topical application. As a rule, these compounds do not irritate the skin and their absorption may therefore remain undetected until systemic symptoms appear, despite the fact that they produce local cholinergic symptoms such as sweating, pilomotor activity, vasodilation, and fasciculations in muscles underlying the area of absorption. All of these symptoms may be explained by local inhibition of cholinesterases. Percutaneous absorption is a relatively slow process, which suggests that there may be considerable inactivation of OPC, particularly of readily hydrolyzable substances, before they reach the blood stream. Enzymes are present in the skin capable of hydrolyzing OPC (5). It seems likely that the marked differences between percutaneous and intravenous toxicities are at least partly due to such detoxifying mechanisms. Absorption is slowed down by the stratum corneum. Minor injuries to the skin reduce this resistance to absorption and may result in a considerable increase in toxicity. In intact skin there may also be transfollicular absorption of OPC. Exposure levels that are toxic but too low to threaten life produce a variety of signs and symptoms in humans, including miosis, muscular fasciculations, and apprehension (2,6). These acute manifestations are usually accompanied by marked desynchronization of the electroencephalogram (7). At higher dose levels, OPC can produce convulsions, muscular paralysis, and death (2). Symptomatic recovery from OPC exposure is normally completed within 2 to 9 weeks, at which time the erythrocyte

cholinesterase level usually has returned to normal (8). There seem to be no qualitative differences in gross symptomatology between percutaneous administration and slow, continuous intravenous infusion; the sequence in which the various symptoms appear seems to be the same and there are no differences in gross anatomical pathology. Sustained low-level administration of OPC can produce symptoms and signs not seen following single exposures to the same dose level. For example, subjects given daily injections of diisopropylfluorophosphate (DFP) showed the additional symptoms of insomnia, excessive dreaming, visual hallucination and tremor (9). However, these symptoms usually disappear shortly after cessation of exposure (9,10).

Biochemical alterations underlying the signs and symptoms of OPC poisoning are sketchy and remain to be defined. Stahl et al (11) reported that shortly after administration of DFP or paraoxon several lysozomal enzymes are affected only slightly, but there is an increase in the activity of plasma beta-glucuronidase. Subsequently, Mandell and Stahl (12) found that the elevation of beta-glucuronidase in the plasma was liver dependent and unaffected by puromycin pretreatment. Thus, the pool of hepatic glucuronidase serves as precursor to plasma enzyme. Since glucuronidase response to OPC is not altered by atropine nor mimicked by neostigmine, it appears that OPC act via some mechanism other than elevation of acetylcholine levels. Other reports (13-15) show that DFP inhibits protein synthesis in the spinal ganglia and in cultured neuroblastoma cells and reduces the activity of liver testosterone hydroxylase.

Animals have a capacity to adapt to sublethal levels of OPC. Rats, for example, adapt to reduced levels of acetylcholine esterase when treated chronically with octamethyl pyrophosphoramide (16, 17), 0,0-diethyl-S-2(ethyl thio-ethyl)-phosphorodithioate (18), DFP (19), and paraoxon (20), and are thus able to tolerate exposure of several times the acute $LD_{50}$ dose over a prolonged period. The adaptive process is unknown but may involve changes in the presynaptic mobilization and storage of acetylcholine (21). Alternatively, there may be enough functional acetylcholinesterase present for the animal to survive or the functional pool of the enzyme may be preferentially recovered in these animals. More recently Sterri et al (22) found that injections of sublethal doses of soman in rats led to tolerance of chronic $LD_{50}$ doses which were markedly higher than the acute dose (22). It has been proposed that the tolerance to soman may be linked to the storage of the lipid soluble compound in adipose tissue or binding of soman to plasma or liver proteins. Thus, storage depots may bind soman before it reaches the acetylcholinesterase in different tissues and, on subsequent gradual release from the depots soman, may be rapidly hydrolyzed so that only small quantities of soman reach the acetylcholinesterase in the nervous tissue.

It is evident from the foregoing observations that metabolic alterations induced by OPC in the skin and other tissues are not fully understood. Consequently, there is a demand for more complete metabolic data on the toxic effects of OPC as well as the metabolic factors involved in the protective reactions against such substances. Due to logistical and administrative problems it was not be possible to use agents such as soman or other nerve agents; instead DFP, a readily available organophosphate, was used as a model compound for more toxic substances. DFP belongs to the same class of cholinesterase inhibitors as soman or sarin and exhibits an identical type of toxicity via the same mechanism (2). In fact, most experimental work is accomplished on DFP and extrapolated to the more toxic agents.

Accordingly, in the present study we determined the effect of topically applied DFP on glucose metabolism in the skin, liver and adipose tissue. Glucose was selected because it is ubiquitous in its distribution and is metabolized throughout the body. The carbon atoms from glucose can be incorporated into most metabolic pools and the pathways are well known.

## METHODS

Adult male rats weighing approximately 350 gm were used in this study. Prior to experimentation the mid-lumbar region of the back was closely clipped and an area of 2x2 cm was outlined on the skin. DFP was mixed with peanut oil (100 mg/ml) and using an Eppendorf microsyringe, four 100 µl aliquots of the DFP solution were successively spread over the outlined area. In this fashion 400 µg of DFP was applied over a 4 $cm^2$ area of the skin. Control animals were treated with four 100 µl aliquots of peanut oil. Visual observations indicated that the skin readily absorbed the quantity of peanut oil applied to the skin. No apparent signs of DFP toxicity were observed. The rats were decapitated two hours following the application of DFP or peanut oil. The central portion of the treated skin plus the liver and epididymal fat pads were excised and cooled in ice-cold saline. The skin was finely minced with a pair of scissors and liver slices were prepared with a Stadie-Riggs microtome, whereas the fat pads were bisected laterally. After being weighed on a torsion balance, approximately 100-mg samples of each tissue were incubated in 25 ml Erlenmeyer flasks containing 3.0 mg of Krebs-Ringer bicarbonate buffer (pH 7.4) and 30 µmoles of glucose with 0.25 uCi of U-$C^{14}$-glucose. The flasks were gassed for 1 minute with 95% $O_2$ : 5% $CO_2$ and stoppered with a rubber stopper from which a plastic well was suspended. All flasks were incubated in a Dubnoff shaker for 2 hours at 37 C at 100 strokes/minute. At the end of the incubation periods 0.2 ml of hyamine hydroxide was introduced in the plastic well by means of a 20-gauge, 2.5 cm needle inserted through the rubber stopper. In addition, 0.5 ml of 1 M sulfuric acid was

injected into the incubation medium and incubation was continued for an additional 60 minutes. The liberated $^{14}CO_2$ was absorbed in the hyamine solution. The plastic wells were removed and placed directly into scintillation vials containing 10 ml of aqueous scintillation counting solution (ACS). The tissues were taken out of the incubation flasks, rinsed several times in normal saline and placed into test tubes containing 10 ml of chloroform and methanol (2:1). The tubes were stoppered and the tissues were extracted overnight. The tissues were then removed from their respective extraction flasks and the lipid extracts were washed three times with "salty" wash as described by Folch et al (23). The washed samples were next saponified for 1 hour at 60 C in 6 ml ethanolic KOH (3 gm KOH in 95 ml ethanol and 5 ml water). Nonsaponifiable lipids were removed by extraction with petroleum ether followed by evaporation to dryness under nitrogen in scintillation vials. The remaining saponified lipids were acidified with HCl and partitioned between water and petroleum ether. The fatty acids in the petroleum ether fraction were placed in scintillation vials and evaporated to dryness under nitrogen. A 0.2 ml aliquot of the aqueous fraction was used to measure glyceride-glycerol-$^{14}C$. All the foregoing $^{14}C$ lipid fractions were dissolved in 10 ml of aqueous scintillation counting solution (ASC) to allow measurement of their radioactivity in a Packard scintillation spectrometer.

Tissue glycogen was isolated from the defatted skin, liver slices or fat pads by digestion in hot 30% KOH followed by precipitation and washing in ethanol. After centrifugation, the supernatant layer was discarded and the glycogen was dissolved in 2 ml of hot water. One ml aliquots were transfered to scintillation vials, 10 ml of ACS fluid containing 4 gm of Cabosil(R) (Packard Instrument Comp) were added and radioactivity determined as above. The results were expressed as nanomoles of glucose carbon incorporated into each metabolite per gram of tissue in one hour. There were eight rats in each treatment group. The significance of difference between means was established by the Student t test. Differences between means were considered significant when $P<0.05$.

RESULTS

The data obtained in this study are summarized in Table 1. DFP had no effect on glucose oxidation or glycerol synthesis in any of the tissues examined. Compared to the controls, DFP decreased glycogen synthesis in the liver by approximately 66% and by about 46% in adipose tissue. DFP had no effect on glycogen synthesis in the skin. DFP stimulated fatty acids synthesis in the skin by approximately 70% and in the liver and adipose tissue by 56 and 92%, respectively. When compared to the corresponding controls DFP doubled and tripled the synthesis of nonsaponifiable lipids, in the skin and adipose tissue, respectively, and enhanced the synthesis in the liver by 69%.

TABLE 1

EFFECT OF DIISOPROPYLFLUOROPHOSPHATE ON GLUCOSE METABOLISM IN THE RAT SKIN, LIVER AND ADIPOSE TISSUE

| METABOLITE | TREATMENT | TISSUE SKIN | LIVER | ADIPOSE |
|---|---|---|---|---|
| $CO_2$ | Control | 3,740±220* | 10,120±1,130 | 401±51 |
| | DFP | 3,440±300 | 9,130±980 | 334±33 |
| GLYCOGEN | Control | 80±34 | 620±48 | 95±15 |
| | DFP | 62±28 | 210±60+ | 49±9+ |
| FATTY ACIDS | Control | 230±25 | 290±25 | 240±25 |
| | DFP | 390±35+ | 450±65+ | 460±18+ |
| GLYCERIDE-GLYCEROL | Control | 440±35 | 480±30 | 111±10 |
| | DFP | 450±52 | 450±45 | 165±18 |
| NONSAPONIFIABLE LIPIDS | Control | 209±38 | 180±20 | 63±8 |
| | DFP | 430±52+ | 350±35+ | 195±31+ |

*Values are nanomoles glucose utilized/gm tissue/hr, mean ± SE from eight rats.

+Indicates significant difference from control values, $P<0.05$, same metabolite, same tissue.

## DISCUSSION

Glucose metabolism in the cell begins with the phosphorylation of glucose to form glucose-6-P. The latter can be metabolized via three metabolic routes. By the Embden - Meyerhof pathway, glucose-6-P, via fructose-6-P and fructose-1,6-diP, is cleaved to produce two triose phosphate molecules, dihydroxyacetonephosphate and glyceraldehyde-3-P. The former can be converted to glycerol via $\alpha$-glycero-P and the latter to pyruvate and then to lactate or the intermediates and products of the tricarboxylic cycle. In the cycle some of the carbon can be oxidized to $CO_2$. Pyruvate, via acetyl CoA and acetoacetate, can be used in the synthesis of fatty acids, cholesterol, and other sterols. In the second route, glucose-6-P can be metabolized via the pentose cycle. In the cycle, glucose-6-P is irreversibly decarboxylated. Lastly, the phosphate groups from glucose-6-P can be transferred to carbon 1, and the glucose-1-P formed reacts with uridine triphosphate to form uridine diphosphate glucose. The glucose residue is then transferred from the nucleotide onto the end of an uncompleted chain in the glycogen molecule to increase the length of this chain by one glucose residue. This reaction, considered the rate-limiting step for glycogenesis is catalyzed by the enzyme glycogen synthetase. The cleavage of the sugar-phosphate bond provides the driving force for the synthesis of the 1,4 glucosidic bond. As the chain length of the polymer is increased to between 12 and 18 residues, the branching enzyme is activated and a glycosal residue is transferred from the 1,4 to the 1,6 linkage. Glycogen metabolism is under control of a complex and dual reciprocal enzyme cascade system involving glycogen synthetase and phosphorylase. Both enzymes are interconverted by a phosphorylation - dephosphorylation reaction sequence involving separate phosphoprotein kinases and phosphatases (24). The biological polarity is reversed in the two enzyme systems; that is phosphorylation activates phosphorylase and inactivates synthetase, whereas dephosphorylation inactivates phosphorylase and activates synthetase. In the process of phosphorylation the terminal phosphate of ATP is covalently bound by esterification with the hydroxyl group of a serine residue.

DFP and other cholinesterase inhibitors react covalently with the serine hydroxyl in the active site of the enzyme, preventing the enzymatic hydrolysis of acetylcholine. A number of other serine enzymes, in addition to acetylcholinesterase are inactivated by reaction with DFP. Among these are chymotrypsin, trypsin, alkaline phosphatase, phosphoglucomutase, and others (25). A marked reduction in glycogen synthesis by DFP observed in our study suggests that the organophosphate affects the activity of glycogen synthetase, the key enzyme in the synthetic pathway. The mechanism by which DFP affects the activity of glycogen synthetase may be similar to that proposed for serine enzymes. As shown in Table 1, DFP did not significantly alter glycogen synthesis in the skin. This may be be due to the large

standard errors in the two treatment groups. Our study shows that DFP has no effect on glucose oxidation. The experimental techniques employed in this study did not allow us to determine the relative contributions of the tricarboxylic acid cycle and the pentose phosphate cycle to the total production of $CO_2$.

Lipid synthesis occurs via a series of reactions involving the conversion of glucose into pyruvate, followed by the intramitochondrial formation of acetyl-CoA. Since acetyl-CoA cannot diffuse into the cytoplasm for fatty acids synthesis, it condenses with oxaloacetate to form citrate. Citrate passes into the cytoplasm where the citrate cleavage enzyme transforms it into extramitochondrial oxaloacetate and acetyl-CoA for lipid synthesis. The extramitochondrial oxaloacetate can be converted into malate via NAD-malate dehydrogenase and then into pyruvate via NADP-malate dehydrogenase in the cytoplasm. An important aspect of these pathways is the formation of NADPH for the reductive synthesis of fatty acids via the coupling of the two malate dehydrogenases. Since the synthesis of long-chain fatty acids requires large amounts of NADPH, additional NADPH is provided by glucose oxidation through the pentose phosphate pathway involving glucose-6-P and 6-phosphogluconate dehydrogenase. As DFP in our study did not stimulate glucose oxidation, it appears that the enhanced fatty acids synthesis is the result of an increased flux of glucose carbons over the fatty acid synthetic pathways. However, other regulatory mechanisms could be involved. Prominent among these are various metabolites and long-chain fatty-acyl-CoA or convalent modification of the key enzymes through a phosphorylation-dephosphorylation cycle.

The synthesis of nonsaponifiable lipids is initiated by the condensation of acetyl-CoA and acetoacetyl-CoA to form 3-hydroxymethylglutaryl-CoA. In the cytosol, hydroxymethylglutaryl-CoA reductase converts the product to mevalonate, a precursor of cholesterol. This is the rate-limiting step in cholesterol synthesis. The various positions of the cholesterol molecule can be enzymatically hydroxylated leading to the eventual formation of pregnenolone and other steroids. Essential elements in the regulation of cholesterogenesis and steroidogenesis are complex and not fully understood. Cellular compartmentalization between the cytosol and mitochondrial turnover of the key enzymes plus substrate induction and feedback inhibition could be some of the regulatory mechanisms in the synthetic pathways. The kinetic form of the key enzymes may also be changed by interaction with other proteins. Such interactions may modify the enzyme's activity for substrate, its velocity, or its capacity for regulation by allosteric modifiers. The precise mechanism by which DFP enhances cholesterol synthesis is unknown. At present, the best understood action of DFP is the phosphorylation of specific intracellular proteins. Phosphorylation of various proteins, both membrane-bound and soluble,

will result in enzyme activation, enzyme deactivation, alterations in m-RNA, alterations in enzyme synthesis, and changes in membrane permeability, depending on the specific protein that is phosphorylated. Before any or all of these possibilities can be determined, more work on the intracellular protein kinases and their substrates will be required.

## CONCLUSIONS

Topical application of DFP induces marked alterations in glucose utilization in the tissues examined, i.e. liver, skin, and adipose tissue. A decrease in hepatic and adipose tissue glycogen synthesis appears to be due to the reduction of the glycogen synthetase activity. Enhanced synthesis of lipids appears to be substrate-induced. However, other biochemical mechanisms may account at least in part, for DFP effects. These may include enhanced glycogenolysis and gluconeogensis, changes in the synthesis of various cofactors, and hormonal interactions.

## RECOMMENDATIONS

- The effects of DFP on the activity of hepatic synthetase-phosphorylase complex needs to be evaluated.

- Metabolic effects of organophosphates on muscle intermediary metabolism should be examined.

- The mechanism by which DFP enhances synthesis of various lipid components needs to be determined.

- The effect of DFP on the flow of glucose carbons via the Krebs cycle and the phosphate shunt should be described.

- DFP effects on glycogen synthesis in the skin need to be reevaluated.

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Research Triangle Park NC 27709

Biological Sciences Division
Office of Naval Research
Arlington VA 22217

Director of Life Sciences
USAF Office of Scientific Research (AFSC)
Bolling AFB
Washington DC 20332

Director
Walter Reed Army Institute of Research
Washington DC 20012

Commander
US Army Medical Research Institute
of Infectious Diseases
Fort Detrick, Frederick MD 21701

Commander
US Army Research Institute
of Environmental Medicine
Natick MA 01760

Commander
US Army Institute of Surgical Research
Brooke Army Medical Center
Fort Sam Houston TX 78234

Commander
US Army Medical Bioengineering
Research and Development Laboratory
Fort Detrick, Frederick MD 21701

Commander
US Army Aeromedical Research Laboratory
Fort Rucker AL 36362

Commander
US Army Research Institute
of Chemical Defense
Aberdeen Proving Ground
Edgewood Arsenal MD 21010

Commander
Naval Medical Research Institute
National Naval Medical Center
Bethesda MD 20014

Commander
USAF School of Aerospace Medicine
Aerospace Medical Division
Brooks Air Force Base TX 78235

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